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FULL RESEARCH PAPER

Lack of biocontrol capacity in a non-pathogenic mutant of *Fusarium oxysporum* f. sp. *melonis*

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Abstract The aim of this study was to assess the biocontrol capacity of rev157, a non-pathogenic mutant of a pathogenic strain of *Fusarium oxysporum* f. sp. *melonis* (Fom24). Inoculated in association with the virulent parental strain, the mutant rev157 did not protect the host plant (muskmelon) against infection by Fom24. Applied on flax, a non-host plant, the mutant rev157 was not able to protect it against its specific pathogen *F. oxysporum* f. sp. *lini*. On the contrary the parental strain Fom24 did protect flax as well as a soil-borne biocontrol strain (Fo47). Since the mutant rev157 was affected neither in its growth in vitro nor in its capacity to penetrate into the roots, it can be speculated that the mutation has affected traits responsible for interactions within the plant. In *F. oxysporum* the pair of strains Fom24/rev157 is a good candidate to identify genes involved in the biocontrol capacity of *F. oxysporum* and to test the hypothesis of a link between capacity to induce the disease and capacity to induce resistance in the plant.

Keywords Biological control · Pathogenicity · Competition · Induced systemic resistance

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Introduction

The fungal species *Fusarium oxysporum* is ubiquitous in soils worldwide. Plant pathogenic strains of this species inducing tracheomycosis or wilts in many crops of economical importance have been well studied for both their ecological behaviour in soil and their interaction with the host plant. They are characterized by their ability to enter the plant roots, to colonize the xylem vessels and from there to colonize the entire vascular system of the plant, inducing symptoms of yellowing and wilting of the leaves. The pathogenic strains show a narrow host-specificity. Indeed, a given strain only infects a single plant species, and many different pathovars, *formae speciales*, have been described. Interestingly, when inoculated to a non-host plant some pathogenic strains can protect the non-host plant against infection by strains belonging to the *forma specialis* specific to the plant species in question (Matta 1966; Mas et al. 1969; Wymore and Baker 1982; Kroon et al. 1991; Huertas-González et al. 1999). For example, strains belonging to different *formae speciales* of *F. oxysporum* were used to induce local and systemic resistance in watermelon (Biles and Martyn 1989). Similarly, non-pathogenic races of *F. oxysporum* f. sp. *melonis* protected muskmelon plants against pathogenic races of the same *forma specialis* (Mas et al. 1969; Huertas-González et al. 1999).

The mechanisms by which soil-borne non-pathogenic strains, or pathogenic strains applied to a

non-host plant, protect the plant are diverse and not fully known. They involve direct antagonism such as competition for nutrients and root colonization between the protective and the pathogenic strain and indirect antagonism expressed through the plant defence reactions. Since competitive interactions contribute to the protection conferred by the biocontrol strains of *F. oxysporum* (Fravel et al. 2003), it has been proposed that a non-pathogenic mutant of a given *forma specialis* would be a good candidate for controlling the disease on the same host plant (Freeman and Rodriguez 1993). Indeed, a non-pathogenic mutant that would have kept all the properties of the pathogenic strain, except pathogenicity, might compete better with the pathogen in the rhizosphere and within the plant tissues than a soil-borne non-pathogenic strain, most probably differing from the pathogen by many traits.

Freeman et al. (2002) produced mutants of *F. oxysporum* f. sp. *melonis* by UV mutagenesis and checked them for pathogenicity on muskmelon and for their ability to protect the plant against the pathogenic parental strain. Two mutants were affected in pathogenicity, and the only mutant completely non-pathogenic was able to significantly reduce mortality of melon and watermelon challenged with their respective pathogens. Transposon mutagenesis has been used to produce non-pathogenic mutants of *F. oxysporum* f. sp. *melonis* (Migheli et al. 2000). Only two mutants had totally lost their pathogenicity (rev 127, and rev157). These mutants offer new opportunities for testing the hypothesis that non-pathogenic mutants are suitable candidates for biological control.

In this study, the mutant rev157 was used to assess the capacity of a non-pathogenic mutant of *F. oxysporum* f. sp. *melonis* to protect the host plant against the parental pathogenic strain (Fom24). This mutant rev157 was also inoculated to flax, a non-host plant, to assess its capacity to protect it against its specific pathogen *F. oxysporum* f. sp. *lini*. The biocontrol capacity of rev157 on flax was compared to that of its parental strain Fom24 and to the efficacy of the soil-borne protective strain Fo47. To ensure that the mutant rev157 was not affected in a trivial vital function such as conidial germination or root penetration, its growth and colonization behaviour was studied in vitro and *in planta.*

Materials and methods

Fungal strains and inoculum preparation

The fungal strains used in this study were: (i) a strain of *F. oxysporum* f. sp. *melonis* (Fom24, race 1-2Y) isolated from a diseased muskmelon from the southern part of France and a mutant of this strain (rev157) changed in its pathogenicity (Migheli et al. 2000); (ii) a pathogenic strain of *F. oxysporum* f. sp. *lini* (Foln3) isolated from a diseased flax plant in the Brittany region, (iii) a non-pathogenic strain of *F. oxysporum* Fo47 isolated from a suppressive soil from the Châteaurenard region (Bouches du Rhône, France) effective in reducing the incidence of Fusarium wilts (Alabouvette et al. 1993; Olivain et al. 1995; Fuchs et al. 1997; Larkin and Fravel 1999).

Fungal strains were cryopreserved as suspensions of microconidia in 25% (v/v) glycerol at −80°C. Frozen conidia were transferred on potato dextrose agar (Sigma-Aldrich, Saint Quentin Fallavier, France) slants in tubes and grown for 7 days at 25°C. Conidia were harvested by washing the surface of the culture with 5 ml of sterile distilled water (SDW). One ml of conidial suspension was transferred to 150 ml of a minimal liquid medium (Correll et al. 1987) in which sucrose was replaced by glucose (5 g l^{-1}) and sodium nitrate by ammonium tartrate (1 g l^{-1}). The fungal strains were cultivated at 25°C on a rotary shaker at 125 rpm (INFORS, AJ 110, Switzerland). After 5 days of growth, fungal cultures were filtered through a sterile number 2 sintered glass funnel (40–100 µm pore size mesh) to retain the mycelium. The microconidia left in the filtrate were harvested by centrifugation (5,000 × *g* for 20 min at 15°C) and washed in SDW. The concentration of the conidial suspension was estimated under the microscope using a haemocytometer, and adjusted to the concentration needed, using SDW.

Bioassays

Growth substrate, culture conditions and inoculation procedures were adapted to each host-pathogen model for optimizing plant growth and appearance of typical wilt symptoms. Plants were watered every day, and once a week, water was replaced by a 500-fold dilution of a commercial nutrient stock solution ('Hydrodrokani AO', Hydro Agri, Nanterre, France).

Plants showing characteristic symptoms of yellowing were recorded once or twice a week and removed. Observations continued until diseased plants were >90% of the diseased control. In each bioassay, there were non-inoculated control plants, plants inoculated with each fungal strain alone and plants inoculated with a mixture of the potential biocontrol strains and the pathogen.

Muskmelon bioassay

Heat-treated (100°C for 1 h) calcinated clay (Oil Dri US special, Terragreen, Brenntag Bourgogne, Montchanin, France) was distributed in 400 ml plastic pots and one muskmelon seed cv. Jerac susceptible to *F. oxysporum* f. sp. *melonis* race 1–2Y was placed in each pot. Muskmelons were raised in a growth chamber, the growing conditions in which were 8 h 23°C N/16 h 25°C D, with a light intensity of 33 μE m^{-2} s^{-1} for 7 days. Each seedling was inoculated with 15 ml of a conidial suspension. The concentrations of the conidial suspension were adjusted to obtain the following inoculum densities: 1×10^3 conidia ml^{-1} of clay for the pathogenic strain Fom24, 1×10^5 conidia ml^{-1} of clay for the mutant rev157 and the biocontrol strain Fo47. From week 2, the temperatures were kept at 18°C N/22°C D. For each treatment there were three replicates of five independent pots with one plant per pot, in a random block design, and the experiment was replicated.

Flax bioassay

A heat-treated (100°C for 1 h) silty-loam soil from Dijon (35.1% clay, 47% loam, 15.1% sand, 1.22% organic C, and 0% $CaCo_3$ [pH = 6.9]) was added to module trays containing 96 wells each of 50 ml. To prevent contamination between treatments, only every second row of wells was filled with soil. The soil in each well was inoculated with 5 ml conidial suspension. The concentrations of the conidial suspensions were adjusted to obtain the following inoculum densities: 1×10^3 conidia ml^{-1} of soil for the pathogenic strain Foln3, 1×10^5 conidia ml^{-1} of soil for the mutant rev157, the parental strain Fom24 and the biocontrol strain Fo47. The soil surface was covered with a thin layer of calcinated clay granules (Oil Dri Chem-Sorb, Brenntag Bourgogne, Montchanin, France) and three seeds of flax, cv. Opaline, were sown in each pot. A thin layer of Chem-Sorb was used to cover the seeds. Plants were grown in a growth chamber; in the first 2 weeks, the growing conditions were 8 h 15°C N/16 h 17°C D, with a light intensity of 33 μE m^{-2} s^{-1}. The plants were thinned to one plant per pot, and from week 3, the temperatures were kept at 22°C N/25°C D. There were three replicates of 16 individual plants per treatment randomly arranged and the experiment was replicated.

Statistical analyses

To compare the ability of strains to induce disease or to protect the plant against wilt, ANOVA was performed on Area Under the Disease Progress Curve (AUDPC) followed by Newman and Keuls test for $P = 0.05$.

Fungal growth and root colonization

Germination of conidia

To compare the ability of the microconidia to germinate, a suspension of conidia prepared as described above was inoculated into minimal liquid medium at a concentration of 1×10^5 conidia ml^{-1}. For each treatment 2 ml of conidial suspension were distributed into six wells of a microtiter plate, and incubated at 25°C. For determination of % germinated conidia, samples of 5 µl were taken from each well, every 2 h, from 4 h until 10 h. Conidia observed under the microscope were considered as germinated when the length of the germ tube was as long as the conidium itself.

Hyphal growth

To compare the fungal growth rate on minimum agar medium, 4 mm diam plugs were taken at the margin of a 7 day-old culture and transferred onto fresh minimal medium. The diameters of the colonies were measured every day for 1 week. There were 10 replicated plates per treatment.

Root colonization

Growth conditions for muskmelon and flax were identical to those of the bioassays, with the only

exception that muskmelons were grown in potting soil instead of calcinated clay. Seedlings were inoculated when they were 7 days old by watering the soil surface with a suspension of conidia in order to get 1×10^5 conidia ml^{-1} soil. At each sampling time, five plants were taken; the soil adhering to the roots was gently removed by washing under tap water, and finally rinsed in SDW. Then they were dried between two sheets of filter paper, and weighed before being surface-sterilized in hydrogen peroxide (5%) for 5s followed by rinsing in SDW three times. The roots were then ground in a mortar for 15s. SDW was added (10 times the root weight) in order to prepare a suspension of root tissues. The suspension was diluted in SDW before being plated on malt extract (10 g l^{-1} Biokar diagnostic, Beauvais France) agar (10 g l^{-1} Biokar diagnostic, Beauvais France) amended with citric acid (250 mg l^{-1}), penta-chloro-nitrobenzene (0.94 g l^{-1} Sigma chemical St Louis USA), chlortetracycline (50 mg l^{-1} Sigma chemical St Louis USA) and streptomycin sulphate (100 mg l^{-1} Sigma chemical St Louis USA). Sampling times were 7, 14, 21, 28, 35, 42 days after melon inoculation and 3, 5, 7, 10 and 13 days after flax inoculation.

Results

Biocontrol capacity

Muskmelon bioassay

When inoculated alone on cv. Jerac the pathogenic strain Fom24 induced symptoms as soon as 9 days after inoculation, and all the plants were diseased 21 days post-inoculation. On the contrary, the mutant rev157 did not induce any symptom on muskmelon plants. When co-inoculated with the pathogen, in the inoculum ratio 1/100, rev157 did not significantly modify the disease progress curve in comparison to the pathogenic strain. In comparison, the non-pathogenic strain Fo47, used as a biocontrol agent, induced a clear decrease in disease incidence (53% of wilted plants compared to 100% in the control). The ANOVA performed on AUDPCs indicated that these differences were significant according to Newman and Keuls test for $P = 0.05$ (Table 1) (Fig. 1a).

Table 1 Muskmelon bio-assay: values of AUDPCs corresponding to the disease progress curves shown in Fig. 1a

Treatment	Mean AUDPC	Newman-Keuls ($P = 0.05$)
Fom24	1360.0 ± 34.6	A
Fom24 + rev 157	1126.6 ± 125	A
Fom24 + Fo47	586.6 ± 191.4	B

There were three replicates of five independent plants. ANOVA was performed on AUDPCs followed by the Newman-Keuls test. Figures followed by the same letter are not significantly different at the probability of 95%

Flax bioassay

When co-inoculated on flax with the pathogenic strain Foln3 in the inoculum ratio 1/100 the strain Fom24 significantly reduced disease incidence: 49 days post-inoculation % diseased plants was 60% in comparison to 97% in the diseased control. This disease reduction was similar to that obtained with the biological control strain Fo47 (68% diseased plants). The ANOVA performed on AUDPCs indicated that these differences were significant according to Newman and Keuls test for $P = 0.05$ (Table 2). On the contrary, the mutant rev157 did not greatly affect the disease progress curve in comparison to plants inoculated with the pathogenic strain Foln3 (Table 2). This experiment has been performed with similar results (data not shown) with flax cv. Viking less susceptible to disease than cv. Opaline (Fig. 1b).

Growth and colonization abilities

On minimum medium the mutant rev157 did not show a significant difference in growth ability compared with the parental strain Fom24. The microconidia germinated at the same rate, as those of Fom24; about 90% showed a germ tube after 10 h of incubation at 25°C (Fig. 2a). The hyphal growth was also similar to that of the parental strain Fom24, and the diameter of the colonies was not significantly different (Fig. 2b).

The ability of the mutant rev157 to colonize the root and the hypocotyl of muskmelon grown in disinfested soil was compared to that of the parental strain Fom24. Results presented Fig. 3a show that 1 week after inoculation Fom24 and rev157 had

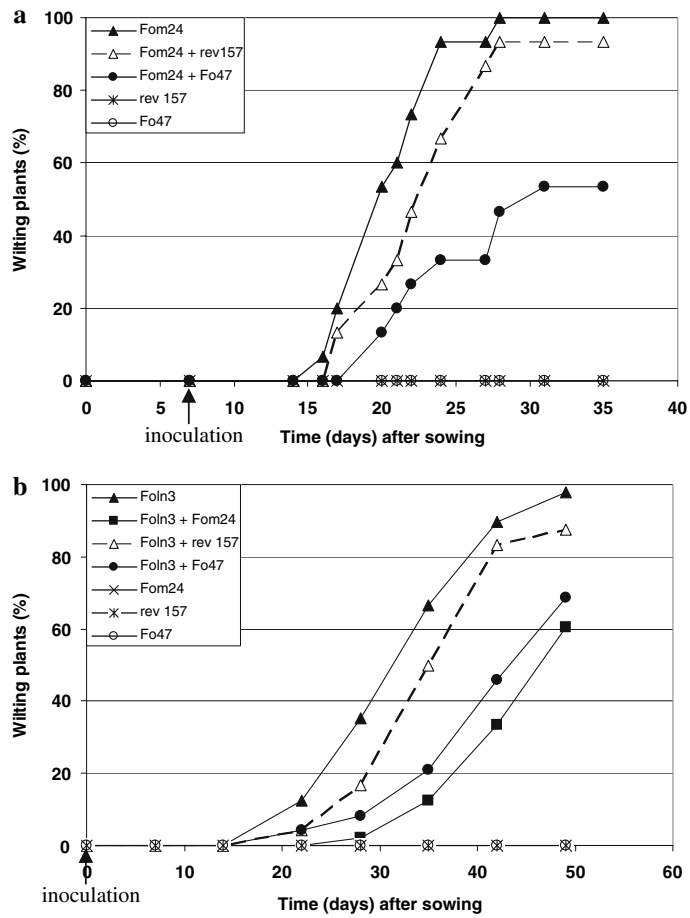

Fig. 1 Capacity of mutant rev157 of *F. oxysporum* f. sp. *melonis* Fom24 and of biocontrol strain Fo47 to protect muskmelon and flax against their respective pathogens. The pathogenic strains were inoculated at a concentration of 1×10^3 conidia ml^{-1} substrate and the non-pathogenic at a concentration of 1×10^5 conidia ml^{-1} substrate, alone or in association. Results are expressed as % wilted plants. (**a**) muskmelon inoculated by the pathogenic strain *F. oxysporum* f. sp. *melonis* Fom24, its mutant rev157 and the biocontrol strain Fo47. (**b**) flax inoculated by the pathogenic strain *F. oxysporum* f. sp. *lini* Foln3, *F. oxysporum* f. sp. *melonis* Fom24, its mutant rev157, and the biocontrol strain Fo47

Table 2 Flax bio-assay: values of AUDPCs corresponding to the disease progress curves shown in Fig. 1b

Treatment	Mean AUDPC	Newman-Keuls ($P = 0.05$)
Foln3	1143.7 ± 46.2	A
Foln3 + Fom24	247.9 ± 145.6	C
Foln3 + rev 157	830.2 ± 250.7	B
Foln3 + Fo47	404.1 ± 96.3	C

There were three replicates of 16 independent plants. ANOVA was performed on AUDPCs followed by the Newman-Keuls test. Figures followed by the same letter are not significantly different at the probability of 95%

colonized the root to the same extent of 270–300 CFU g^{-1} fresh weight of root tissues. The concentration of the pathogen then increased to 5×10^3 CFU g^{-1} 42 days after inoculation. The colonization of rev157 however decreased and stabilized at 50 CFU g^{-1}. Rev157 was not detected in the hypocotyl, whereas the pathogen Fom24 reached the concentration of 5×10^4 CFU g^{-1} of hypocotyl tissues, 35 days after inoculation.

A similar experiment was conducted with flax, to compare the ability of the strains Fom24 and the mutant rev157 to colonize the root tissues of a non-host plant. Both Fom24 and rev157 colonized flax roots and their colonization did not differ. Their densities ranked from 0.5 to 1.2×10^3 CFU g^{-1} 3 days post-inoculation, and from 4.9 to 7.7×10^3 CFU g^{-1} (fresh weight of root tissues) 10 days after inoculation for Fom24 and rev157, respectively (Fig. 3b). The same range of colonisation was detected in flax root tissues colonized by Fo47 (data not shown).

Discussion

In a previous study, we produced non-pathogenic mutants of Fom24, a pathogenic strain of *F. oxyspo-*

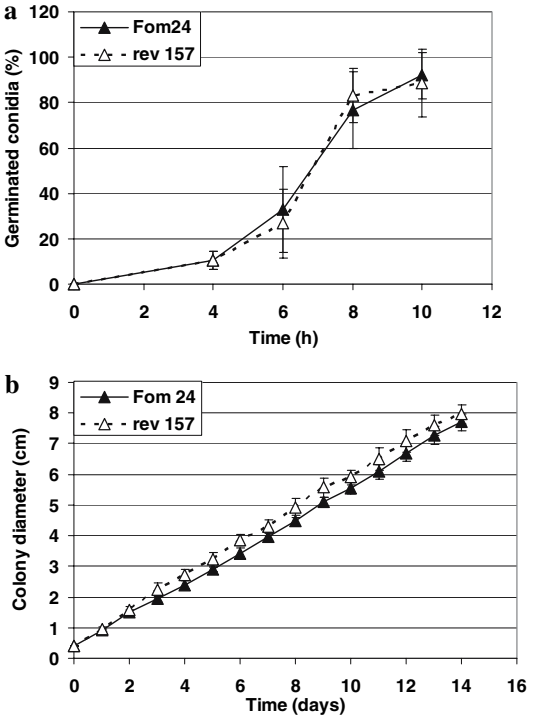

Fig. 2 Saprophytic growth in vitro of the mutant rev157 and the wild-type strain *F. oxysporum* f. sp. *melonis* Fom24. (**a**) ability of microconidia to germinate; minimal liquid medium was inoculated with 1×10^5 conidia ml^{-1} and incubated at 25°C. (**b**) hyphal growth expressed as diameter of the colony of fungi on minimal agar medium inoculated with an explant of 4 mm

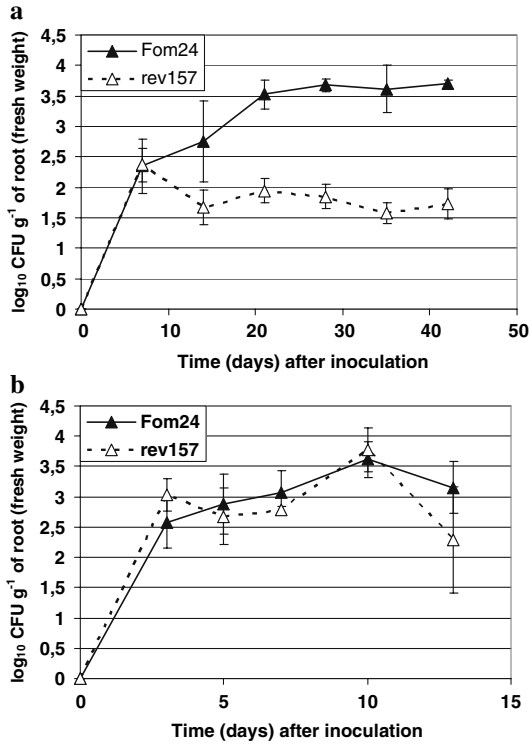

Fig. 3 Root colonization by the mutant rev157 and the wild-type strain *F. oxysporum* f. sp. *melonis* Fom24 expressed as log CFU g^{-1} of root tissues. The fungi were inoculated at a concentration of 1×10^5 conidia ml^{-1} soil. (**a**) muskmelon, (**b**) flax

rum f. sp. *melonis* (Migehli et al. 2000). One aim of the present study was to assess the biocontrol capacity of one of these non-pathogenic mutants: rev157. Results clearly showed that rev157 that had lost the capacity to induce wilt in muskmelon and was not able to protect the host plant against infection by the pathogenic parental strain Fom24. This loss of pathogenicity and lack of biocontrol capacity on the host plant are not related to deficient traits regarding the growth behaviour of this strain either in vitro or in soil. The non-pathogenic mutant rev157 was detected in the root tissues, indirectly showing that conidia inoculated into the soil had been able to germinate in the rhizosphere followed by penetration of the hyphae into the root tissues. The difference in population density in the root tissues may be related to the inability of the mutant to reach the vessels. On the contrary, the parental pathogenic strain established in the vessels, and grew towards the hypocotyl where it was detected. Finally it induced symptoms on the leaves 21 days after inoculation. The inability of the mutant rev157 to colonize the stele was confirmed by microscopic observations of transverse root sections (data not shown) suggesting that the loss of pathogenicity could be related to the inability of this mutant to establish in the vessels.

In contrast to results published by Freeman et al. (2002) who isolated a non-pathogenic mutant of *F. oxysporum* f. sp. *melonis* able to protect muskmelon, rev157 did not protect the host plant when co-inoculated with the parental strain. Rev 127, another non-pathogenic mutant of Fom24 was also unable to protect the host plant. Therefore non-pathogenic mutants derived from a given pathogen do not necessarily present a biocontrol capacity, as has been suggested in the case of *Colletotrichum magna* (Redman et al. 1999) and *Fusarium oxysporum* (Freeman et al. 2002) based on the assumption that ''non pathogenic mutants may compete better than their original pathotype''.

Knowing that a pathogenic strain of *F. oxysporum* applied to a non-host plant is able to protect it against its specific *forma specialis* (Biles and Martyn 1989), the second aim of this study was to determine whether the non-pathogenic mutant rev157 retained the capacity of the parental strain to protect a non-host plant species. Results showed that rev157 was not able to decrease disease incidence on flax although the parental strain Fom24 did control the disease as efficiently as the protective strain Fo47. The biocontrol capacity of Fom24 is in agreement with results obtained by Matta (1966) who used a strain of *F. oxysporum* f. sp. *melonis* to protect tomato against *F. oxysporum* f. sp. *lycopersici*. This phenomenon initially described as cross-protection or premunition (Matta 1971) is considered today as an expression of induced systemic resistance (Van Loon 2000). Soil-borne protective strains of *F. oxysporum* are usually effective through an association of modes of action including competition for nutrients in the rhizosphere, competition for root colonization and induced resistance (Alabouvette et al. 2007). Our results show that rev157 has not been affected in its growth behaviour either in vitro or in planta since it colonizes the flax root to the same extent as the protective parental strain. Moreover, the level of root cortex colonisation observed for rev157 was comparable to that reported earlier for the soil-borne protective strain Fo47 (Olivain and Alabouvette 1997; Benhamou and Garand 2001). This suggests that the lack of biocontrol capacity of the mutant rev157 is not related to its parasitic growth in the root tissues but to its inability to induce plant defence reactions sufficiently to prevent disease development.

We can therefore speculate that the mutant rev157 has been affected in its ability to induce resistance of the plant. These results which suggest the role of induced resistance in the plant do not contradict previous results demonstrating the role of competition between pathogenic and protective strains. Indeed, introduction of rev157 in mixture with the pathogenic strain Foln3 always induced a slight reduction of disease incidence. This effect might be due to competitive interactions in the rhizosphere.

Interestingly, these results reveal a connection between pathogenicity on the host plant and biocontrol capacity on a non-host plant, since the mutant that had lost its pathogenicity on muskmelon had also lost the biocontrol capacity on flax. We can speculate that the fungal genes affected by the mutation are involved in the mechanisms of recognition of the fungus by the plant leading to the disease in a host plant and to induced resistance in a non-host plant. This hypothesis has to be further examined and invites us to study the physiological events induced in cell cultures inoculated by these fungi to determine whether the production of active oxygen species or ion fluxes (Olivain et al. 2003) would enable us to differentiate the pathogenic strain from its non-pathogenic mutant on muskmelon and the protective strain from its non-protective mutant on flax. Moreover, this pair of strains, Fom24/rev157, constitutes a good model to identify genes involved in the biocontrol activity of *F. oxysporum*. Since it has not been possible to tag genes by transposon mutagenesis (Migheli et al. 2000) we are planning to utilize a subtractive hybridization technique to reveal genes expressed during the protective interaction with the plant.

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